Protocol for work with the flow-chamber biofilm-system

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1) Assembly of flow channels

Biofilms are cultivated in flow channels.

The flow channels are cut/drilled in plexiglass, covered with an object glass to allow inspection with a microscope. A flow cell is assembled by adding a thin layer of silicone between the channels (sausage-like), without touching the plexiglass with the syringe. Put a glass cover slip on top of the silicone and push carefully until the silicone covers the whole area of plexiglass between the channels (Figure 1). Allow the silicone to dry before use (preferably over night).

Silicone tubings with dimensions 1 mm inner diameter, 3 mm outer diameter are connected to each end of the flow channel.

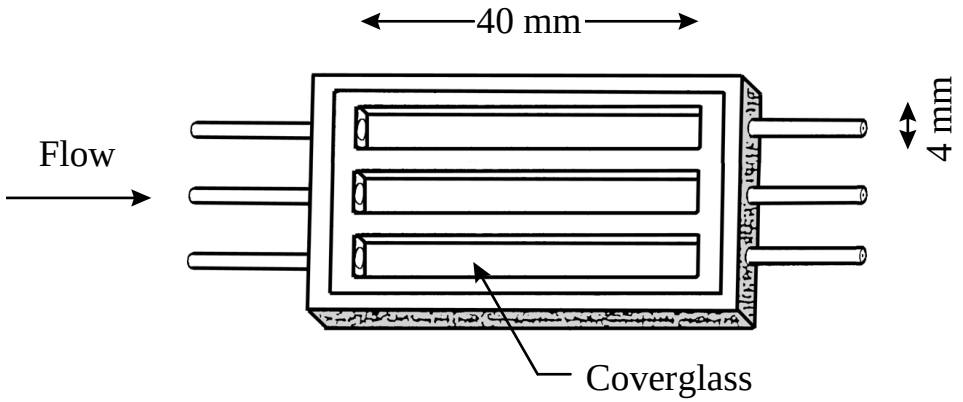

Figure 1: 3-channel flow cell.

2) Assembly of flow system

Except for the tubes going through the pump, the flow system is assembled using silicone tubing. For the pump we use the much stronger Marprene tubing. Media bottle, bubble trap and flow-channels are connected with tubings as shown in Figure 2. The bubble traps are 5 mL syringes mounted on the bubble trap base with inlet and outlet (the inlet holes are situated higher than the outlet holes) inside the bubble trap. The syringes are closed in the top with a stopper.

The effluent media is collected in bottles containing 2,5% CETYL. The effluent-bottles must be placed at, or above the level of the flow channels. In this way a leak in a flow channel will result in media running out of the leak and not air into the leak, which therefore is more sterile, and do not create bubbles that can destroy the biofilm.

Hints:

- It's possible to seal leaks in the system by adding silicone and at the same time drying with paper tissue.
- Place the bubble traps in small trays during the preparation of the system.

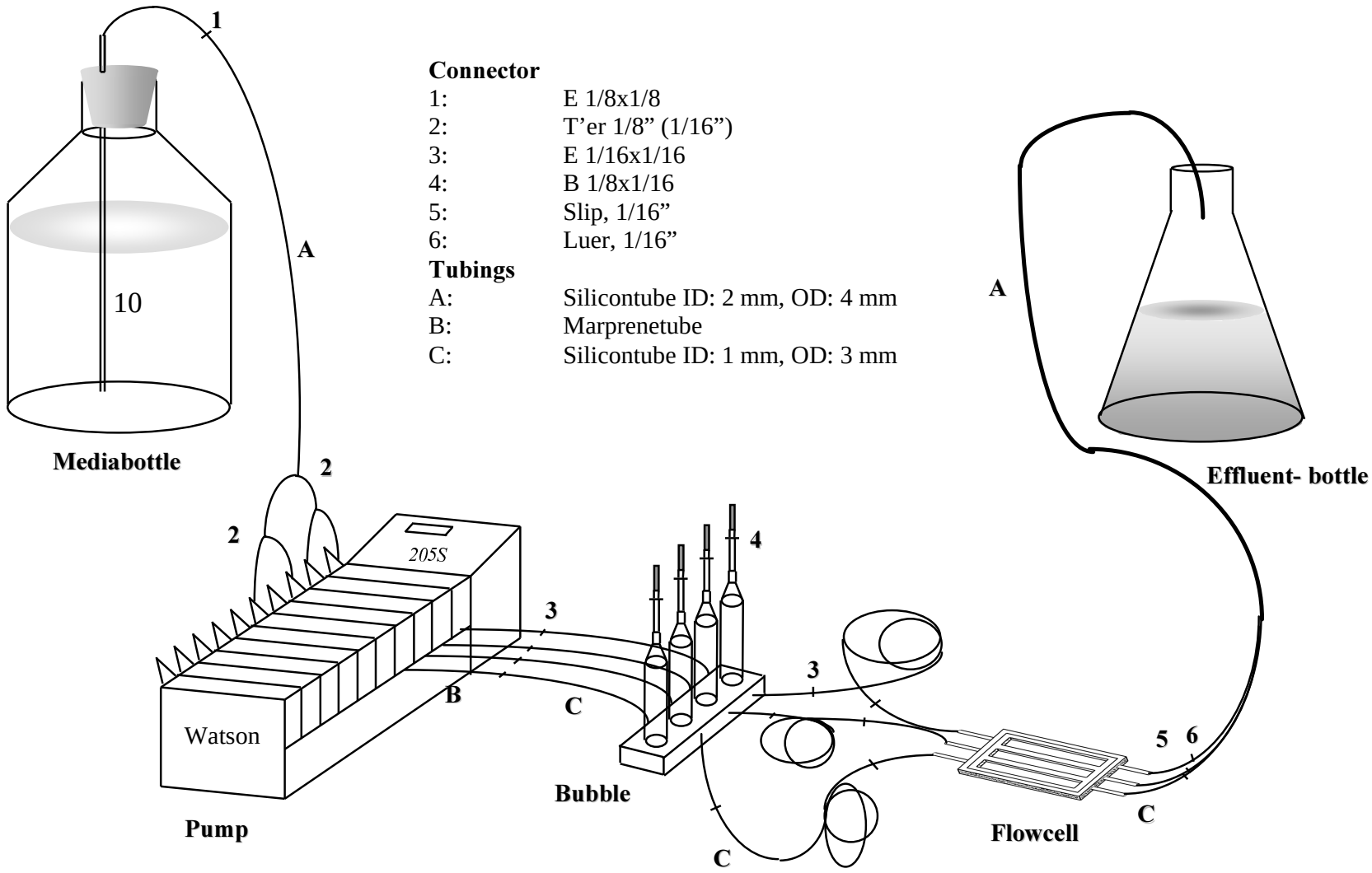

Figure 2: Biofilm-system setup

3) Preparing the flow system

Procedure for filling the flow system

Start to fill the flow system, by taking off the stoppers on top of the bubble traps (keep the stopper sterile by putting them in 96% EtOH).
When the bubble traps are filled, put on the stoppers again to fill the rest of the flow system (flow channels and effluent tubing). Make sure that all air-bubbles are out of the system by carefully tapping the flow cells while the pump is running (5-10 min.).
To empty the system, just disconnect the bottle with either water or media or hypochloride-solution and let the system run at high speed (90 rpm).

Sterilization of the flow system

Sterilization of the flow system is done by pumping 0.5% hypochlorite (33 mL 15 Vol % Sodiumhypochlorite in 1 L of sterile H_2O) into the system and allow to sterilize for 3-4 h.

Washing the flow system

The flow system is washed to remove the hypochlorite. This is done by filling and emptying the system 2-3 times, as described in "Procedure for filling the flow system", with 1-2 L of autoclaved H_2O depending on the amount of flow channels.

Running media through the flow channels

The flow system is filled with media as described and the flow rate is setted to approximately 0.2 mm/s. This is obtained by setting up the 12-channel Watson Marlow pump at 2.00 rpm and the 16-channel Watson Marlow pump at 1.75 rpm.

Hint:
- If the carbon source is very hydrophobic and/or you are going to run your experiment at 30°C or 37°C it is advisable to fill the system with the media and let it run overnight at the certain temperature to saturate the silicone tubing before inoculation.

4) Inoculation of flow channels

Before each inoculation, the flow is stopped and the tubing between the flow channel and the bubble trap is clamped off.
The tubing before the inlet is sterilized with 96% ethanol. A 0.5 mL syringe with needle is filled with 250 µL of an overnight culture of the cells (diluted 10-1000 times or to a specific OD

depending on the experiment. Dilute in ABT or 0.9% NaCl). The syringe needle is inserted in the tubing as near as possible to the flow channel inlet and the cells are carefully injected into the channel. After inoculation the tubing is sterilized with 96% ethanol, and the injection hole is closed by adding a thin layer of silicone. To allow the cells to establish on the glass surface turn the flow cells with the glass surface upside down. After 1 hour turn the flow cell back, take of the clamps and start the media flow.

Hint:
- It is possible to inoculate more cells later, but make sure to inject the cells very carefully.

5) Procedure for using the Zeiss LSM 510 Meta

When using and before leaving the CLSM room, remember:

- Don't change or install anything on the computer
- Don't update the computer
- Don't change the temperature in the CLSM room
- Do never turn off the Microscope separately
- If you are going to use the heating table, ask somebody that knows about it
- Turn off the lamps if not in use
- When turning off the CLSM, turn off the lasers and then close the program
- Wait 15 min before shutting down the computer and the remote control
- Don't turn off the microscope at any time
- Don't touch the microscope or the computer if you have oil on your fingers
- Clean objective and xy-table with ethanol before leaving the CLSM
- Don't leave things in the CLSM room

- **If you are in doubt about something, please ask!**

Starting up the Microscope-system

Turn on the equipment in the following order:

1. Remote Control
2. Computer (button on the keybord)

Wait for Windows to start.

- Press "Ctrl+Alt+Del" when prompted.
- Log in with name and password.

The computer is now ready for use.

- Open "LSM 510" by pressing the icon.

A window LSM510 Switchboard appears on the screen:

- Press "Scan new images".
- Press "Start expert mode".

A window LSM510 expert mode appears on the screen.

- Press "Acquire".

Now you can choose between a number of buttons. By pressing these, you will open the different windows.

- Open the window Laser Control by pressing the button "Laser".
- Turn on the laser units you are going to use:
    - *Argon/2* for CFP, GFP, YFP, SYTO9 and reflection
    - *HeNe1* for RFP, PI etc.
    - *HeNe2* for CY5, SYTO62 etc.
    - *Titanium:Saphire* for 2-photon scanning

NOTES: - The Argon/2 laser unit should be OFF or STANDBY when not used for more than 15 minutes.

- Before you close down the whole system, allow the lasers to cool down for 15 minutes.

- Open the window Configuration Control by pressing the button "Config".
- Press the icon "channel mode"; a new window Track Configurations appears.
- Choose between “Single Track” and “Multi Track”, depending on the number of colours you want to detect. (E.g. “Single Track” for gfp or “Multi Track” for a mixture of yfp + cfp)

- Choose your configuration from the Configuration List by pressing the icon “Config” and press “Apply” (do never press store here as you will overwrite saved configurations.)

Viewing the specimen with the microscope

- Upper right pin should be in when viewing.
- Turn on/off the light by pressing the "HAL" button on the right side of the microscope.
- Open the window Axioplan Control by pressing the button "Micro".

You can change objectives, filter settings etc. of the microscope here. But you can change these also manually on the microscope (two buttons on right site of the scope under the focus nub).

- Change filters on the screen (window Axioplan Control, section "Reflector Turret"), or use the buttons on the left side of the microscope, just under the "focus nub".
    - None (Filter DIC) for normal light microscopy
    - Fset MF13f/r Filter5 for GFP, YFP... and filter 6 for Rfp, PI…

  Or use the buttons on the left side of the microscope, just under the "focus nub".
- The button "FL" (above "HAL") turns on and off the UV lamp.

Scanning the specimen

- Upper right pin should be out when scanning.
- Start by pressing the button "Scan" in the window LSM510 expert mode.

The window Scan Control appears.

- If you are going to do a Z-scan: Press "Z scan" in the upper part of the window
- Press “Z scan” again to take a scan a single layer.
- Press "find" in the right part of the window or find the cells manually using the focus nub.

The computer will now make a pre-scan while finding the 'best' settings for laser and detector. You can adjust these manually for each channel under the topic "Channels" in the upper part of the window Scan Control.

HINT: You can also open an image previously saved and press the "reuse" button; then you'll get the settings that were used for that particular image.

- Press "Z settings".
- Press "Fast XY".

The specimen will be scanned continuously now. By turning the focus nub of the microscope towards yourself, you will find the 'top' of the specimen.

- Press "First".

Then turn the focus nub the other way until you find the 'bottom' of the specimen.

- Press "Last".
- Press "Stop".

The specimen is now ready to be scanned.

- Select how many layers you want to scan by typing the number in "num slices".
- In Mode select the scan speed and the quality of the image by choosing 1, 2, 4 or 8 in the averaging box (1 give a pure fast image, 4 a good quality image)
- Start the scanning by pressing "Start".

Saving images

- A) To create a new Database: Go to "File" and than "New". A window will open and you should type in the name of your experiment etc. Press the icon "Create"

  B) In case you have a database already, just press the icon "Save as" from the window of your scanned picture and be aware of, that it saves it in YOUR database.

The images will be saved as .lsm file and can be treated afterwards using "Imaris" software.

If you want to save the images as .tif files:

- Open the image you want to save.
- Press "file" in the window LSM expert mode.
- Press "export".
- Choose "raw data series" and "TIF - tagged image file".
- Select the folder and save the image.

Closing down the system

- Bring the object table of the microscope in upper position.
- Turn off all the laser units.
- Close all open windows.
- Wait 15 minutes after you turned off the lasers.
- Shut down the computer; it usually turns off automatically.
- Turn off the Remote Control.

- Clean the microscope oil objective with ethanol on lines paper and cover the microscope with the blue cap.

6) Media for biofilmwork

Original biofilm media

- **A-10**

	final conc. in media:
20 g $(NH_4)_2SO_4$	15,1 mM
60 g $Na_2HPO_4 \bullet 2H_2O$	33,7 mM
30 g KH_2PO_4	22,0 mM
30 g NaCl	0,051 M

Fill up to 1 L with H_2O (pH should be 6,4 ± 0,1) and autoclave.

- **FB:**

9 L Milli-Q H_2O
10 mL 1 M $MgCl_2$
1 mL 1 M $CaCl_2$
1 mL Trace metals (for *P. aeruginosa*-biofilm)
or 1 mL 10.000x Fe-EDTA (1,83 g in 50 mL H_2O) for *E. coli* etc.

autoclave

- Mix **1 liter A-10 + 9 liter FB** and add the **carbon source.**